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In vitro and in vivo interleukin-8 production in human renal cortical epithelia

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In vitro and in vivo interleukin-8 production in human renal cortical epithalia. The signals resulting in leukocytes infiltrating the tubulointerstitial compartment during renal inflammatory disease are not well understood. A recently described cytokine, interleukin-8 (IL-8), has been demonstrated to be chemotactic for lymphocytes and neutrophils at picomolar and nanomolar concentrations, respectively. Cytokeratin positive, renal cortical epithelial cells (RCEC) with tubular attributes were cultured from kidney tissue from six human subjects. We report that these human renal cortical epithelial cells in primary cell culture respond to either IL-1β, TNF or LPS in both a time- and dose-dependent manner by expressing IL-8 mRNA and secreting antigenic IL-8 peptide. In addition, RCEC were found to be strongly positive for cell-associated antigenic IL-8 peptide by immunostaining after 24 hour incubation with IL-1β, TNF and LPS. To ascertain whether IL-8 was present in renal disease associated with infiltrating leukocytes, we performed immunohistochemistry on renal biopsy specimens from patients with acute allograft rejection. Both proximal and distal tubular epithelial cells were found to be strongly positive for cell-associated antigenic IL-8. These findings suggest that the human renal tubule epithelial cell may actively participate in acute inflammatory processes in the kidney, including allograft rejection, by effecting and directing leukocyte chemotaxis via the production of IL-8.

Renal interstitial inflammation is a principal cause of both native and allograft kidney failure [1, 2]. A number of inflammatory kidney diseases, including transplant rejection, vasculitis, glomerulonephritis and pyelonephritis, are associated with an interstitial nephritis. The predominant histopathology associated with this lesion is the presence of leukocytes of both mononuclear and polymorphonuclear morphology in the renal interstitium. The mechanism(s) by which cells are attracted into the tubulointerstitial compartment remains to be fully elucidated. Interleukin-8 (IL-8) is a recently described chemotactic cytokine [3] with the potential to contribute in directing immune cell traffic into the renal interstitium.

IL-8, also known as neutrophil chemotactic factor (NCF) and neutrophil activating peptide-1 (NAP-1), was originally described as a secreted product from LPS-stimulated human peripheral blood monocytes that had neutrophil chemotactic and activating activity [3]. This cytokine is a potent, highly stable, soluble chemotactic cytokine at nM and pM concentrations for neutrophils and lymphocytes, respectively [4]. It has also been shown to mediate neutrophil activation at nM to mM concentrations [5]. IL-8 is resistant to many proteases, and its secretion requires de novo synthesis [5]. Thus, unlike short-lived chemotactic factors such as complement fragments or arachadonic acid products, IL-8, with prolonged stability, has the potential for longer duration of action at sites of tissue inflammation. Although initially described as a monocyte product [3], it has recently been shown that IL-8 is expressed in and secreted by non-immune types of human cells. These include primary dermal fibroblasts [6], umbilical vein endothelial cells [7] and the following cell lines of epithelial origin: pulmonary A549 [8] and hepatocyte Hep-G2, SK-Hep and Hep-3B [9].

In this manuscript we demonstrate the capability of human renal cortical epithelial cells isolated from primary culture to both express IL-8 mRNA and secrete IL-8 antigen in response to TNF, IL-1β and LPS. We also provide in vivo data that show that renal tubular epithelial cells produce IL-8 during transplant rejection. These data imply that renal cortical epithelial cells themselves can act as non-immune effector cells in renal inflammation by producing IL-8.

Methods

Cell culture

Human renal cortex-derived epithelial cells were isolated using previously reported techniques [10]. Briefly, 2 cm^3 of human renal cortex was obtained from the non-involved pole at the time of elective nephrectomy for hypernephroma or transitional cell carcinoma, and placed in ice cold, sterile normal saline. The capsule was removed, the medulla dissected away, and the cortex diced in cold sterile saline to form a slurry of approximately 1 to 2 mm pieces. The slurry was transferred into a sterile flask containing 150 to 200 ml 0.25% trypsin (porcine) in phosphate buffered saline and gently stirred for 30 minutes at 37°C. The contents of the flask were then filtered through one layer of sterile gauze and the filtrate was centrifuged at 30 × g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 20 to 30 ml of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% glutamine, 1% streptomycin, 1% penicillin. The mixture was

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placed in a 75 cm^2 polystyrene tissue culture flask (Corning) and maintained at 37°C with 95% air/5% CO_2. After 24 hours of incubation, the media was gently replaced to remove tissue debris and non-adherent cells. After 7 to 10 days the confluent cells underwent first passage. All experiments were conducted on third and fourth passage cells.

Cell characterization

The cultured renal cortical cells were characterized with monoclonal immunostaining. Minimal or no staining was seen for desmin or factor VIII-vWF. All cells showed moderate staining for actin. Bright fluorescence, consistent with intermediate filaments, was seen for all cells using the anti-cytokeratin monoclonal antibody. This monoclonal antibody staining pattern is consistent with a homogeneous primary culture of cells of epithelial origin [11, 12].

The hormonal stimulation of cAMP was used to further delineate the origin of these epithelial cells. Confluent RCEC cultures from two subjects were incubated with 10^{-7} M parathyroid hormone (PTH), 10^{-6} M calcitonin and 10^{-6} M arginine vasopressin (AVP) for 15 minutes. Supernatants were then replaced with a solution containing 150 mM KCl, 5 mM potassium phosphate, 2 mM EDTA and 0.5 mM 3-isobutyl-1-methylxanthine (pH 6.8), and the cells were scraped into this medium and the suspension was then immediately sonicated and boiled for five minutes Samples from both the plate supernatants and cell fraction were stored frozen for measurement of cAMP after acetylation by a standard radioimmunoassay performed by the University of Michigan Diabetes Research and Training Center. Stimulation with AVP failed to increase in cAMP. In the first subject, stimulation of RCEC with PTH and calcitonin resulted in a 3.7- and 1.4-fold increase in cell fraction and 3.7- and 2.2-fold increase in supernatant cAMP over controls, respectively. In the second subject calcitonin stimulation increased cell fraction and supernatant cAMP 1.3- and 3.4-fold over control, respectively. These results are compatible with hormone sensitive, renal epithelial cells of tubule origin [10, 13].

Northern blot analysis

Total cellular RNA from the stimulated renal cortical cells was isolated using a modification of the method of Chirgwin et al [14] and Jonas, Sargent and Davis [15]. Total RNA was separated by Northern blot analysis using formaldehyde in 1% agarose gels, transblotted to nitrocellulose, baked, prehybridized, and then hybridized with a ^{32}P 5′ end-labeled oligonucleotide DNA probe. A thirty-mer oligonucleotide probe complementary for human IL-8 (5′-GTT-GGC-GCA-GTG-TGG-TCC-ACT-CTC-AAT-CAC-3′) was used under conditions we have previously defined as specific for IL-8 [6, 7]. Autoradiograms were quantified using laser densitometry (Ultrascan XS, LXB Instruments, Inc., Houston, Texas, USA). Equivalent loading of total RNA per lane was assessed by ethidium bromide stained 28S and 18S rRNA.

Interleukin-8 ELISA

IL-8 antigen was quantitated using a modified, double ligand ELISA method, as previously described [16, 17]. Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F) were coated with rabbit anti-IL-8 antibody (9.5 μg in 100 μl, 0.1 M $NaHCO_3$, pH 9.6) for 16 hours at 4°C and then washed with phosphate buffered saline, pH 7.5/0.5% Tween-20 (washing buffer). Nonspecific binding sites were blocked with 2% bovine serum albumin in washing buffer and incubated for one hour at 37°C. Plates were rinsed three times with washing buffer and diluted samples (100 μl) in duplicate were followed by incubation for 120 minutes at 37°C. Plates were washed three times, biotinylated rabbit anti-IL-8 (final concentration 1:2000) was added, and plates were incubated for 90 minutes at 37°C. Plates were washed three times, then streptavidin-peroxidase conjugate (Dakopatts, Denmark) was added and incubated for 30 minutes at 37°C. Plates were washed three times and citrate phosphate-buffered orthophenylenediamine solution (Dakopatts) was added and incubated for approximately five minutes at room temperature to the desired extinction. The reaction was then terminated with 3 M H_2SO_4. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant human IL-8, from 1000 ng to 0.05 pg per well. This ELISA method consistently detected IL-8 concentrations above 50 pg/ml.

Immunohistochemistry

Immunolocalization of IL-8 antigen was performed by fixing renal cortical cells grown on 8 chamber LabTek (Nunc, Inc., Naperville, Illinois, USA) slides in 4% paraformaldehyde in PBS for 10 minutes, then rinsing twice with PBS. Prior to staining, the slides were again fixed for 15 minutes in 1:1 absolute methanol and 3% H_2O_2, rinsed in PBS, and nonspecific binding sites blocked with a 1:50 dilution of normal goat serum. Normal serum was removed, followed by the addition of either normal rabbit serum (1:2000 dilution) or rabbit antihuman IL-8 serum (1:2000 dilution). After 15 minutes of incubation at 37°C, the slides were rinsed with PBS, overlaid with biotinylated goat antirabbit IgG (1:200; Vector Laboratories), incubated 15 minutes, and rinsed two times with PBS. The slides were treated with Streptavidin conjugated to peroxidase for 15 minutes at 37°C, rinsed three times, over laid with substrate chromogen (3-amino 9-ethylcarbazole) for seven minutes at 37°C to allow color development, and rinsed with distilled water. Mayer's hematoxylin was used as counterstain. Processing of human renal allograft material was performed as follows. Formalin fixed, paraffin embedded sections were cut from a needle core biopsy of a human renal allograft and placed on polylysine-coated plastic slides. The sections were deparaffinized with xylene wash × 2, rehydrated using stepwise increasing water/ethanol concentrations, and then placed in PBS. Thereafter immunohistochemical staining for IL-8 was done as described above, except for the substitution of an avidin bound phosphatase-chromogen system (Vector). Antibody specificity was demonstrated by competitive inhibition studies, in which recombinant IL-8 when preincubated with the anti-IL-8 antibody, resulted in complete inhibition of immunostaining. In addition, IL-8 affinity purified anti-IL-8 showed similar results to that of non-affinity purified anti-IL-8 antibody.

Reagents

Reagents used in this study were: DMEM (Whittaker, Walkersville, Maryland, USA), trypsin, glutamine, penicillin, streptomycin (Hazleton, Lenexa, Kansas, USA), fetal bovine serum (FBS; Biocell, Carson, California, USA), LPS (*E. coli* 0111:

B4), PTH, and AVP (Sigma Chemical Co., St. Louis, Missouri, USA). Human recombinant IL-1β with a specific activity of 30 U/ng was from the Upjohn Co. (Kalamazoo, Michigan, USA). Human recombinant TNF with specific activity of 22 U/ng was supplied by Genetech (South San Franciso, California, USA). Human recombinant IL-8 was purchased from Peprotech (Rocky Hill, New Jersey, USA). IL-8 antiserum was produced by immunization of rabbits with recombinant human IL-8 in multiple intradermal sites with complete Freund's adjuvant (Sigma) [18]. IL-8 antiserum, in concentrations used in the ELISA or immunohistochemical localization of antigenic IL-8, reacted with human recombinant IL-8 in Western analysis, and failed to cross react with CTAP-III, NAP-2, beta-thromboglobulin, GRO/MGSA or platelet factor 4. Monoclonal antibodies were: aDesmin (Labsystems, Helsinki, Finland), aCytokeratin and aActin (Enzo Diagnostics, Inc., New York, New York, USA), aVWF-factor VIII (Miles, Inc., Kankakee, Illinois, USA), aVimentin (Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA).

Statistical analysis

The individual experiments described were performed in tissue obtained from six human subjects. To accommodate for variability in the absolute response from each subject, the results were expressed as a percent of the maximal response for each subject. Data were analyzed using the Staview II statistical software package (Abacus Concepts, Inc.) run on a MacIntosh II computer (Apple, Inc., Cupertino, California, USA). Data are expressed as means ± SEM. ANOVA with the Scheffe test was used to detect significant differences between the group means.

Human subjects

Human renal tissue was obtained using methods approved by the University of Michigan Hospitals Human Use Committee.

Results

IL-8 gene expression: Dose dependant analysis of steady state IL-8 mRNA

Initial studies were performed to determine whether human RCEC expressed IL-8 mRNA in the presence of either IL-1β, TNF or LPS. These cells were stimulated with IL-1β (0.02 to 20 ng/ml), TNF (0.02 to 20 ng/ml) or LPS (0.1 to 10 μg/ml) for eight hours and then total RNA was isolated ($N = 6$). Control cells were maintained and processed in the same manner but were not stimulated. The Northern blot analysis of the dose-dependent expression of RCEC-derived IL-8 mRNA is shown in Figure 1. A Northern blot from a representative subject is shown in Figure 1A. Laser densitometry of IL-8 mRNA was pooled from a total of six subjects and these data are shown in Figure 1B. Stimulation by IL-1β, TNF or LPS resulted in a 25- ($P < 0.01$), 7- ($P < 0.05$), and 6- ($P < 0.01$) fold increase in steady state IL-8 mRNA over unstimulated control, respectively. In other experiments we have found that as little as 2 pg/ml IL-1β could induce IL-8 mRNA (data not shown). The half maximal induction of steady state IL-8 mRNA was seen with 0.2 ng/ml, 2.0 ng/ml, 10 ng/ml for IL-1β, TNF and LPS, respectively. Although these data indicated that all three proinflammatory stimuli were effective for the induction of RCEC-derived IL-8 mRNA, IL-1β appeared to be the most consistently potent agonist. In separate experiments, we found that either IL-1β, TNF or LPS stimulation in the presence of 100 mM cyclohyeximide resulted in superinduction of RCEC-derived IL-8 mRNA (data not shown).

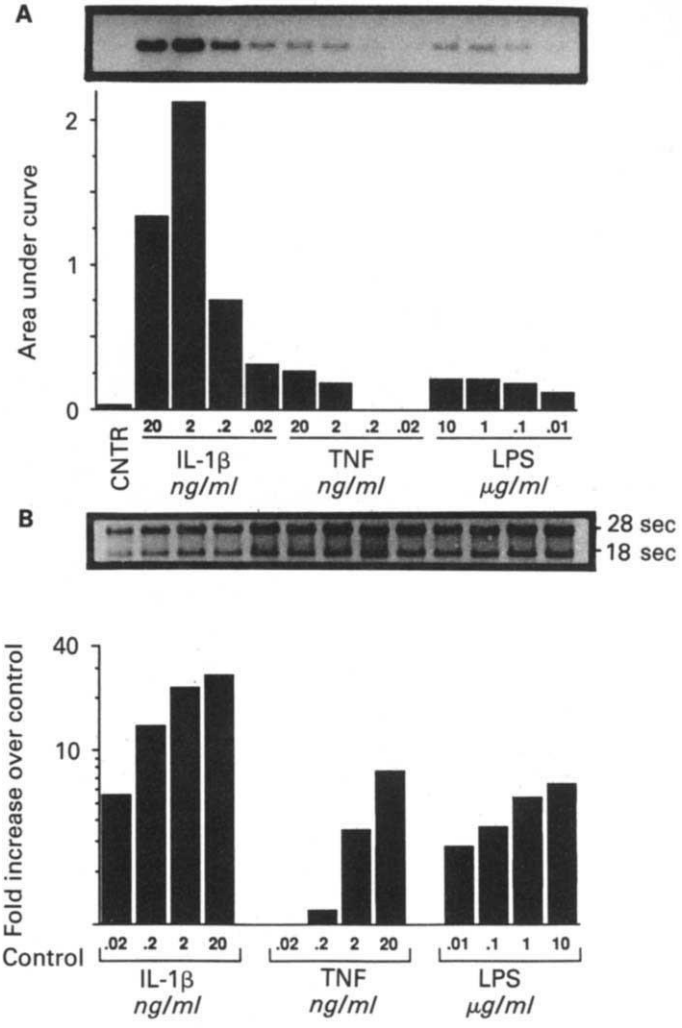

Fig. 1. *Dose dependent expression of steady-state IL-8 mRNA from human renal cortical epithelial cell cultures after proinflammatory stimulation.* **A.** Northern blot with corresponding laser densitometry from a representative subject. The stimuli concentrations are in decreasing order. The 28S and 18S rRNA represents equivalent loading of total RNA. **B.** Mean fold increase over control of steady state IL-8 mRNA for 6 subjects. The stimuli concentrations are in increasing order.

IL-8 gene expression: Time dependent analysis of steady state IL-8 mRNA

To define the temporal expression of IL-8 mRNA after proinflammatory agonist stimulation, the following experiments were performed. RCEC were stimulated with IL-1β (0.2 ng/ml), TNF (2 ng/ml) and LPS (1 μg/ml) for the specified periods of time and total RNA was isolated ($N = 6$). Control cells were not stimulated but were similarly maintained and processed as stimulated cells. The kinetic analysis of steady state levels of

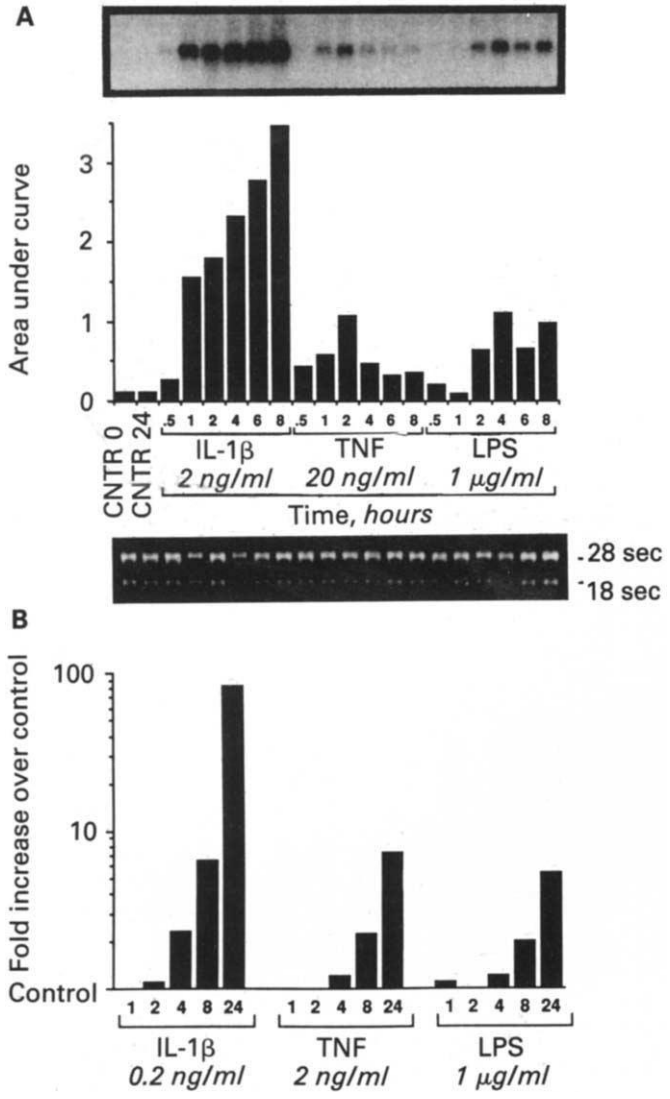

Fig. 2. *Time dependent expression of steady-state IL-8 mRNA from human renal cortical epithelial cell cultures after proinflammatory stimulation.* **A.** Northern blot with corresponding laser densitometry from a representative subject. The 28S and 18S rRNA represents equivalent loading of total RNA. **B.** Mean fold increase over control of steady state IL-8 mRNA for 6 subjects.

RCEC-derived IL-8 mRNA is shown in Figure 2. A representative Northern blot is shown in Figure 2A and a summary of Northern blots from RCEC-derived IL-8 mRNA from six subjects is shown in Figure 2B. In Figure 2A, IL-1β is shown to stimulate the most rapid and greatest expression of IL-8 mRNA. The averaged data from these experiments (Fig. 2B) demonstrate increasing expression of IL-8 mRNA with time after stimulation by IL-1β, TNF and LPS, with maximal expression seen at 24 hours for these agonists. IL-1β resulted in maximal IL-8 steady-state mRNA expression.

Cell associated IL-8 antigen

The results of experiments discussed thus far indicate that RCEC have the capacity to express IL-8 mRNA in both a dose- and time-dependent manner after proinflammatory agonist stimulation. To determine whether IL-8 mRNA was translated into IL-8 peptide, immunocytochemical localization of IL-8 antigen from RCEC was performed using an avidin bound peroxidase (brown pigment). RCEC were stimulated with either IL-1β (2 ng/ml), TNF (20 ng/ml) or LPS (1 μg/ml) for 24 hours, then fixed and immunostained for cell associated IL-8 antigen. As shown in Figure 3, greater than 95% of the RCEC stimulated by either IL-1β, TNF or LPS demonstrated a cytoplasmic distribution of antigenic IL-8 as compared to the negative serum control. Staining for IL-8 antigenic peptide was seen as early as four hours post-stimulation (data not shown).

Extracellular IL-8 antigen

Having shown that IL-8 mRNA was expressed in both a time- and dose-dependent fashion, as well as being translated into cell-associated antigenic peptide after proinflammatory agonist stimulation, we next determined whether either IL-1β, TNF or LPS stimulation resulted in secreted IL-8 antigen. RCEC from six subjects were either stimulated in a concentration or time dependent manner with IL-1β, TNF or LPS. RCEC supernatants were isolated and then assayed for antigenic IL-8 by ELISA. The eight hour control level of supernatant IL-8 in these experiments was 10.9 $\pm$ 4.9 ng/ml. In Figure 4A, secreted antigenic IL-8 is seen to increase in response to increasing concentrations of proinflammatory agonist stimulation. Maximal secretion of IL-8 occurred at approximately 2 ng/ml IL-1β ($P < 0.001$), 20 ng/ml TNF and 1 μg/ml LPS ($P < 0.001$). Half-maximal stimulation was seen at 0.2 ng/ml, 2 ng/ml and 100 ng/ml with IL-1β, TNF and LPS, respectively. As shown in Figure 4B, supernatant IL-8 was detected at approximately two to four hours, four to eight hours, and four to eight hours for IL-1β, TNF and LPS, respectively. For each of the three proinflammatory agonists, maximal supernatant IL-8 occurred 24 hours post-stimulation.

Cell associated IL-8 in acutely rejecting human renal allograft

Since our in vitro studies of human renal cortical epithelial cells demonstrated expression, translation and secretion of IL-8 after stimulation with IL-1β, TNF and LPS, we next determined whether IL-8 had exaggerated expression under conditions of acute human renal allograft rejection. Renal tissue was obtained from an allograft biopsy at day 9 post-transplant from three patients with pathologic diagnosis of acute allograft rejection. Figure 5 shows the immunohistochemical localization of IL-8 antigen using an avidin bound phosphatase (pink pigment) from a representative biopsy specimen. Control sera failed to show non-specific immunostaining (Fig. 5A), however, anti-IL-8 immune sera demonstrated cell associated IL-8 localized to the cytoplasm of both proximal and distal renal tubular cells (Fig. 5B). Normal human renal tissue failed to immunostain for IL-8 (data not shown).

Discussion

In this study we present evidence that human renal cortical epithelial cells, of tubule origin, can express and secrete IL-8, a potent neutrophil and lymphocyte chemotactic peptide. These cells were capable of responding to a primary (LPS) and

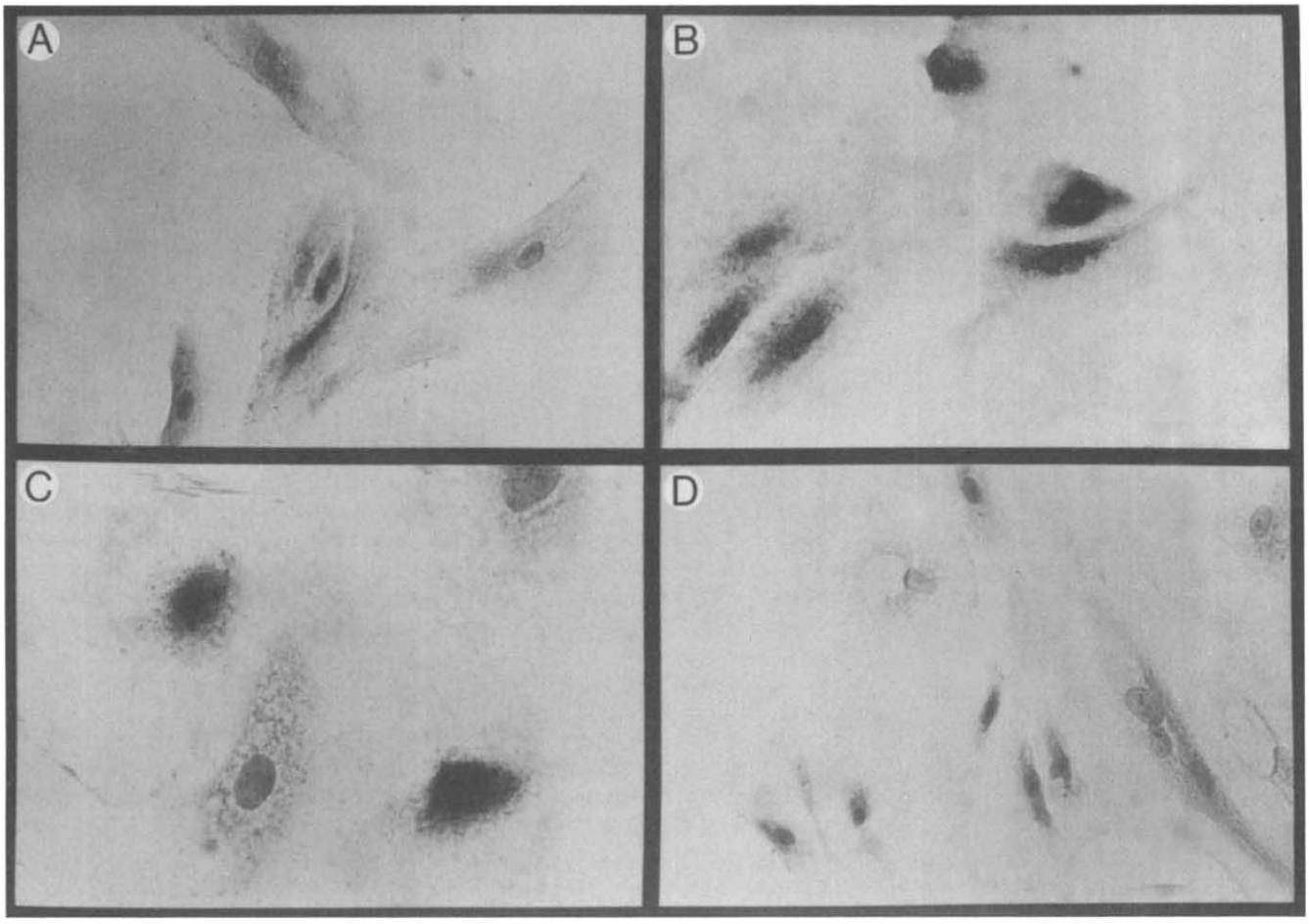

Fig. 3. *Immunoperoxidase localization of IL-8 from human renal cortical epithelial cells after 24 hr incubation with the following stimuli.* **A.** 20 ng/ml TNF; **B.** 2.0 ng/ml IL-1β; **C.** 1.0 μg/ml LPS; **D.** the 24 hour control with preimmune serum.

secondary (IL-1β or TNF) stimulus in both a time- and dose-dependent fashion by the expression of IL-8 mRNA and the production of IL-8 antigen. In addition, both distal and proximal renal tubular epithelial cells from a rejecting human renal allograft were shown to manifest cell-associated antigenic IL-8 peptide. Thus, in the human kidney, recruited inflammatory cells may receive chemotactic information from several sources, including resident interstitial mononuclear phagocytes, newly recruited mononuclear cells and renal tubular epithelial cells. The contribution of the renal tubule epithelial cell in directing inflammatory cell traffic may have particular importance in renal allograft rejection, where the recruitment of leukocytes could be dependent upon activation of allograft tubular epithelial cells. However, other types of tubulointerstitial disease including pyelonephritis, vasculitis and allergic interstitial nephritis could also be mediated, in part, by similar mechanisms. This is one of the first reports of primary epithelial cell cultures from a human organ expressing and secreting IL-8. Recently, IL-8 mRNA expression and protein production has been reported in human retinal pigment epithelia [19].

Both primary (exogenous) and secondary (endogenous) types of proinflammatory agonists mediate RCEC expression and secretion of IL-8. In contrast to other epithelia, LPS can serve as a primary stimulus for the expression and secretion of IL-8 by RCEC, similar to mononuclear phagocytes [3] and endothelial cells [7]. LPS-induced IL-8 production may be important in the setting of acute pyelonephritis, in which the predominant etiologic agent, the gram negative bacterium, is a principle source of LPS. By responding to LPS, renal tubular cells could rapidly induce a host inflammatory response with the generation of IL-8 without need for intermediate immune cell-derived cytokines.

The cytokines IL-1β and TNF are early response, inflammatory cytokines of the immune system. IL-1β is secreted by a variety of cell types including mononuclear phagocytes, mesangial cells, endothelial cells and fibroblasts [20–22]. Stimuli for the induction of IL-1 from these cells includes LPS, C5a, immune complexes interferon-γ, and IL-2 [20, 23, 24]; TNF producing cells are principally mononuclear phagocytes and T-lymphocytes [25, 26]. A variety of stimuli are important for the induction of TNF, depending upon the cell type studied [27, 28]. Both of these polypeptides have pleiotropic effects in a number of cell systems [27]. In a positive feedback fashion, IL-1β can stimulate the expression of its own receptor, thus, functioning at a local level to amplify the inflammatory response [29]. The capacity of renal tubular epithelial cells to respond to IL-1β is evident from several recent reports. IL-1 can modulate glucose and amino acid transport in cultured murine proximal

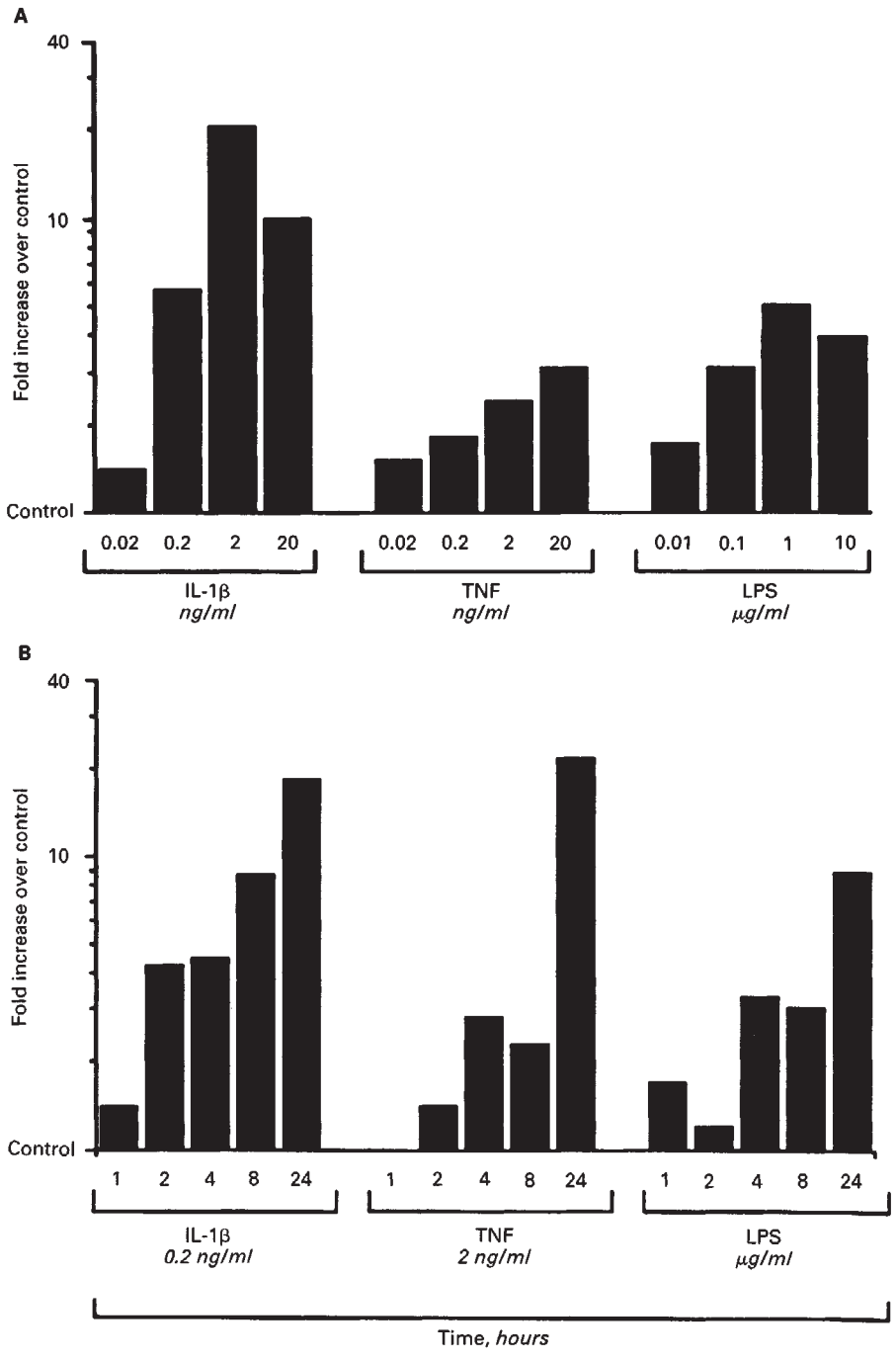

Fig. 4. *The dose* (**A**) *and time* (**B**) *dependent production of antigenic IL-8 from human renal cortical epithelial cell cultures after proinflammatory stimulation.* IL-8 production expressed as the mean fold increase over control ($N = 6$).

tubular epithelium [30], as well as induce naturesis in rats through probable tubular mechanisms [31]. We have shown that IL-1β and TNF can stimulate renal tubular cells to express IL-8 mRNA and secrete antigenic IL-8. By responding to IL-1β and TNF, the renal tubule epithelial cell is not only capable of altering its tubular functions but also able to participate with both monocytes and lymphocytes in a cytokine coordinated network to recruit inflammatory leukocytes.

In the setting of acute rejection of a renal allograft, we have shown immunohistochemical staining for IL-8 in human renal tubular cells. The intense nature of this staining, coupled with the large mass of tubular cells in the kidney, is evidence of the potential of human renal allograft epithelium to participate in acute rejection by establishing an IL-8 chemotactic gradient for leukocytes. In the non-infected renal allograft, the induction of IL-8 in tubular cells is presumably through the production of signals from either host lymphocytes or mononuclear phagocytes that are activated by foreign MHC antigens. These

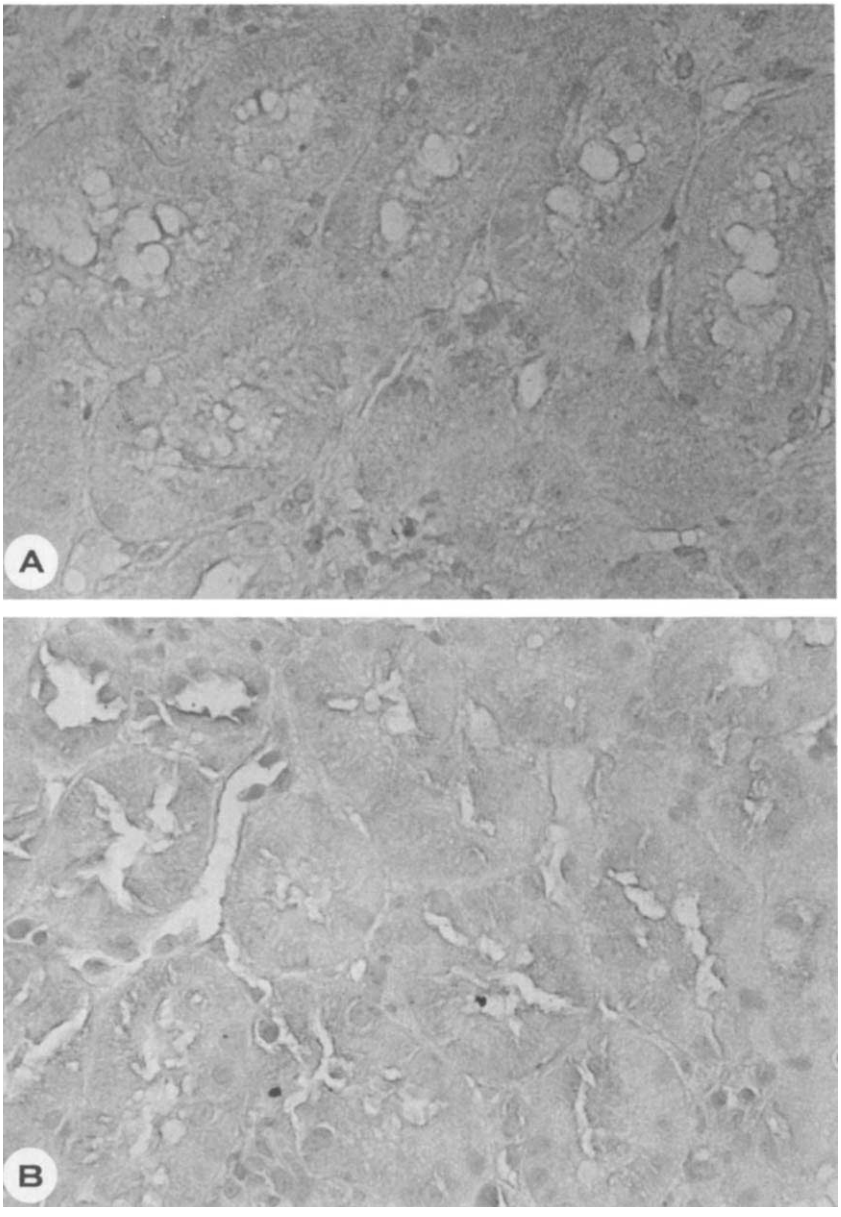

Fig. 5. *Immunohistochemical localization of antigenic IL-8 from a representative biopsy specimen from an acutely rejecting human renal allograft.* **A.** Control with preimmune serum. **B.** IL-8 immune serum.

inflammatory signals likely include IL-1β and TNF, both of which have been implicated as important immune stimuli in acute allograft rejection [32, 33].

These studies further support the role of the human renal tubular epithelial cells as effector cells that are important participants in the initiation and maintenance of renal inflammatory responses.

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